

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 9/16, 9/51	A1	(11) International Publication Number: WO 99/58112
		(43) International Publication Date: 18 November 1999 (18.11.99)

(21) International Application Number: PCT/GB99/01449

(22) International Filing Date: 7 May 1999 (07.05.99)

(30) Priority Data:
9810236.1 13 May 1998 (13.05.98) GB

(71) Applicant (for all designated States except US): MICROBIOLOGICAL RESEARCH AUTHORITY [GB/GB]; CAMR (Centre for Applied Microbiology and Research), Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): FARRAR, Graham, Henry [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). TINSLEY-BOWN, Anne, Margaret [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB). JONES, David, Hugh [GB/GB]; Microbiological Research Authority, CAMR, Porton Down, Salisbury, Wiltshire SP4 0JG (GB).

(74) Agents: SCHLICH, George, William et al.; Mathys & Squire, 100 Gray's Inn Road, London WC1X 8AL (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

With international search report.

(54) Title: IMPROVEMENTS RELATING TO ENCAPSULATION OF BIOACTIVE AGENTS

(57) Abstract

Bioactive agent is encapsulated in a polymer microparticle in a (water-in-oil)-in-water emulsion-based method, and using a solvent that comprises ethyl acetate. Also described are microparticles comprising low inherent viscosity (i.v.) PLG, some with i.v. less than 0.5 dl/g, and methods for their preparation. DNA release is modified through use of low i.v. PLG. A particle production method for scale-up uses a blender that avoids excessive shear damage to DNA being encapsulated.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

- 1 -

IMPROVEMENTS RELATING TO ENCAPSULATION OF BIOACTIVE AGENTS

The present invention relates to improvements in encapsulation of bioactive
5 agents, such as antigens, drugs and DNA for vaccination and gene therapy. In
particular, the present invention relates to methods for encapsulating antigen
and / or DNA, in aqueous solution, in a polymer microparticle, so that the
microparticles when administered to a recipient deliver antigen to antigen
presenting cells of the recipient, and / or induce expression of the DNA in the
10 recipient in the antigen presenting cells. The present invention relates also to
microparticles and compositions comprising microparticles.

The present technology of microencapsulation is currently at least 10 years old
but does not seem to have yielded any commercially successful products,
15 despite many announced breakthroughs. Indeed, apart from WO-A-97/17063
(by the same inventors as the present invention), published methods have been
found to be inefficient and unreliable.

Many published patents and applications are in the name of the Southern
20 Research Institute (SRI). In particular, US-A-5407609 purports to describe in
example 7, an emulsion based method for the manufacture of hollow particles.
Another emulsion based method for making particles that contain a protein,
specifically BSA, is described in Sah *et al* (J Microencapsulation, 1995, vol. 12,
no. 1, pp 59-69). If these particles are to be used as a means of administering
25 an encapsulated bioactive agent to antigen-presenting cells in the gut
epithelium, it is of great importance that their size be below 10 microns in
diameter. Larger particles are not endocytosed by the targeted gut cells and
pass through the gut without effect.

30 However, the methods detailed in US-A-5407609 and by Sah *et al* succeed in
making relatively large particles, or at least particles over a wide range of sizes,
where a significant portion of particles are larger than the biological activity cut

- 2 -

off point of 10 microns. A large spread of particle sizes, such as that seen in US-A-5407609 inevitably leads to much of the encapsulated agent being incorporated in particles of a size that are not appropriate for phagocytosis. Worse still, Sah *et al* obtained a mixture of particle sizes with an apparent
5 minimum diameter of around 10 microns. Both art methods are, therefore, not specifically designed to produce encapsulated product available for uptake by antigen-presenting cells. The encapsulation method used by Sah also requires very high shear rates during encapsulation, shear rates that would have a detrimental effect upon, and are thus unsuitable for, encapsulation of agents
10 such as DNA.

US-A-5407609 makes reference to the construction of microbubble-like particles but emphasises that water soluble agents are not easily encapsulated in such particles due to a tendency for the encapsulated agent to migrate out
15 of that part of the emulsion that will eventually constitute the internal content of the particles. To overcome this problem, US-A-5407609 requires that as soon as the emulsion has formed it be immediately added to a large volume of extraction medium i.e. water. However, in doing so control over particle size may be lost.

20 The internal architecture of microparticles made using the Sah method is generally a honeycombed matrix, illustrated in the photographs that accompany the Sah paper, rather than a simple, hollow microbubble-like structure. Incomplete removal of solvent and/or water from the insides of these matrices
25 during the solvent extraction step and subsequent lyophilisation can result in the premature degradation of the polymer of the particle and a corresponding fall in the pH of any encapsulated aqueous solution, leading to damage of an encapsulated agent.

30 The bioactive agent release profile of administered microparticles has been previously altered by changing the relative amounts of lactide and glycolide present in PLG polymer. Much of this data is however based on "microsphere"

- 3 -

technology where the encapsulated agent is distributed throughout a polymer matrix and there is no known prior art that relates to control of the release profile of microbubble particles. Altering the relative amounts of lactide and glycolide present in the PLG polymer has shown limited success in the short term but it would be desirable to provide alternative means of controlling the release profile.

WO-A-97/17063 describes methods for obtaining significant levels of incorporation of DNA into microparticles however, the authors of the present invention would like to improve upon this incorporation efficiency.

The present invention thus seeks to overcome, or at least ameliorate, the problems observed in the art. In particular, preferred embodiments of the invention aim to provide improved technology for the encapsulation of bioactive agents such as antigen and / or nucleic acids in polymer microparticles for the delivery of antigen and / or gene based prophylactics and therapies.

Accordingly, the present invention provides a method of encapsulating DNA in a polymer microparticle, comprising:

- dissolving polymer in a solvent to form a polymer solution;
- preparing an aqueous solution of DNA;
- combining the polymer and DNA solutions with agitation to form a water-in-oil emulsion;
- adding the water-in-oil emulsion to a further aqueous phase containing a stabiliser or surfactant with agitation to form a (water-in-oil)-in-water emulsion;
- adding the (water-in-oil)-in-water emulsion to excess of an aqueous phase to extract the solvent, thereby forming polymer microparticles of a size up to 10 microns in diameter, said microparticles containing DNA;
- wherein the DNA comprises a coding sequence and induces expression of the coding sequence in a recipient following administration, and wherein the solvent comprises ethyl acetate.

- 4 -

Following this method it is advantageously found that preparations of microparticles are obtainable with DNA being encapsulated in such a way that it retains its ability to induce expression of its coding sequence - as confirmed by transformation and transfection assays using DNA recovered from microparticles. The preparations are suitable for inducing protective immunity in a recipient after oral administration of the DNA coding for an appropriate antigen. The sizes of microparticles are generally less than 10 microns in diameter, thus increasing the proportion of agent available to antigen presenting cells. (Eldridge J.H. *et al*, J. Controlled Release, Volume 11, 1990, pages 205-214). Also, the efficiency of incorporation of DNA into the microparticles is improved and in preferred embodiments of the invention has been found to approach 80%, this latter figure representing a significant advance over the art.

The method of the invention is thus essentially a solvent extraction method, in which extraction of solvent after the second emulsion stage, i.e. after forming the (water-in-oil)-in-water emulsion, solidifies or consolidates the polymer of the microparticles. Reference to "forming polymer microparticles" is intended as a reference to the overall method in which microparticles are initially formed via an emulsion and polymer shells of the microparticles are then solidified or consolidated by solvent extraction.

The solvent used to dissolve the polymer can be extracted from the (water-in-oil)-in-water emulsion in a number of ways. In a specific example of the method of the invention described below, the (water-in-oil)-in-water emulsion is quenched in a large volume (100ml-1l) of warm water (37 degrees celsius is used in the example), thereby facilitating evaporation of the solvent from the polymer into the water. Other examples of solvent extraction are known, and include use of larger volumes of water at room temperature and use of a rotary evaporator.

One method of determining the incorporation efficiency of the method of the invention is to accurately measure, by optical absorption spectroscopy, the

- 5 -

amount of DNA that is released from known weights of microparticles and relating this quantity to the original amount of DNA used. Using this method of optical measurement, it is observed that, in preferred embodiment of the invention, incorporation efficiency typically of 60-70% is achieved, representing
5 a significant advance over the prior art methods.

The DNA contained within the microparticle will typically comprise double stranded DNA. The construction of a suitable DNA sequence for use in the invention will be appreciated by persons of skill in the art and is described in
10 WO-A-97/17063. It is preferred that the sequence comprises both a transcriptional promoter and a gene coding sequence. It is further preferred that the DNA sequence provides for transcription termination and polyadenylation downstream of the coding sequence.

15 It is particularly preferred that the DNA be double stranded, circular and super coiled. It has been observed that during manufacture of microparticles the DNA is subjected to shear forces. Using the particle manufacturing conditions of the invention, the inventors have managed to retain significant amounts of functional DNA, though have observed that previously supercoiled DNA may
20 become partly converted to the open circular form during the process. The degree to which the encapsulation process denatures DNA can be assessed by separating the native (super-coiled) DNA from partially denatured (open circle) and denatured (nicked and degraded) DNA, using agarose gel electrophoresis. A typical example of such a separation is illustrated in Figure 1 where clearly
25 defined bands correspond to the two most abundant form of DNA in the preparations - super-coiled (lower band) and open circular (upper band). Total degradation of the DNA is usually interpreted from a disappearance of bands corresponding to the two common forms.

30 The retention of biological activity of the encapsulated DNA, a measure of the retention of the preferred physical state, can be assessed, following release of DNA from the microparticle, by measuring the capacity of known amounts of

- 6 -

DNA to either transform competent bacteria or transfect eukaryotic cells in culture. The transformation assay measures the introduction of a functional antibiotic resistance gene (e.g. β -lactamase) which is present in the plasmid and which confers ampicillin resistance to susceptible bacteria. Expression of this gene indicates the overall retention of DNA structure. The transfection assay specifically measures the functionality of the plasmid to induce expression of the gene of interest in suitably manipulated cells in culture. In both cases the indexes of activity obtained are compared to the activity of equivalent amounts of corresponding stock DNA.

Plasmid DNA or DNA derived therefrom by conventional manipulations is particularly suitable. As there is extensive literature relating to plasmid manufacture a person of skill in the art will readily be able to prepare a plasmid suitable for the microparticle of the invention. In general, plasmids incorporating any eukaryotic or prokaryotic promoter sequence are suitable.

The polymers most suitable for preparing the microparticles of the invention typically exhibit a number of properties. Such polymers should be of low toxicity, ideally pharmaceutically acceptable and soluble in the solvent adopted, such as in ethyl acetate either with or without a cosolvent, preferably to a level of at least around 50mg/ml. In addition, they are typically biocompatible and biodegradable although it is preferable that they be sufficiently stable to pass through the acid conditions of the stomach. Nevertheless, the invention is not intended in its broadest aspects to be limited to a particular single polymer.

Polymers based on poly (amino acids / derivatives of amino acids) are suitable and some specific examples of these are polymers including poly(lactide), poly(glycolide) and / or poly(lactide - co-glycolide). In a specific embodiment of the invention, described in more detail below, the polymer is poly-(DL lactide-co-glycolide) (PLG), and the concentration of PLG in the polymer solution is typically at least 10% wt/volume. In another specific embodiment of the invention also described below, the polymer is polycaprolactone.

The ratio of lactide to glycolide in the PLG suitable for manufacturing the microparticles of the invention is not critical and commercially available polymers include lactide:glycolide ratios of 25:75, 50:50 and 75:25 though the ratio can suitably be anywhere in the range 0:100 to 100:0. In particularly preferred embodiments of the invention the polymer is PLG of a molecular weight greater than 70kD, or less than 50kD. Other suitable polymer formulations for microparticles according to the present invention include poly-hydroxybutyrate, poly hydroxyvalerate, poly (hydroxybutyrate/valerate), ethyl cellulose, dextran, polysaccharides, polyalkylcyanoacrylate, poly-methylmethacrylate, poly(ϵ -caprolactone), polyhydrazines and mixtures of all of these components.

A further aspect of the invention relates to the universality of the method used to prepare the microparticles. Specifically, the inventors have made microparticles using polymers across a wide range of molecular weights without substantial modification of the basic methodology. Accordingly, the invention provides a method of encapsulating a bioactive agent in a polymer microparticle, comprising:-

- dissolving polymer in a solvent to form a polymer solution;
- preparing an aqueous solution of the bioactive agent;
- combining the polymer and bioactive agent solutions with agitation to form a water-in-oil emulsion;
- adding the water-in-oil emulsion to a further aqueous phase containing a stabiliser or surfactant with agitation to form a (water-in-oil)-in-water emulsion;
- adding the (water-in-oil)-in-water emulsion to excess of an aqueous phase to extract the solvent, thereby forming polymer microparticles of a size up to 10 microns in diameter, said microparticles containing the bioactive agent; wherein the polymer comprises or consists of PLG of molecular weight of 40kD or lower.

Preferably, the molecular weight of the PLG is 30kD or lower, and in specific

embodiments of the invention microparticles comprising PLG of 3kD, 6kD, 9kD, 22kD and mixtures thereof. The molecular weight range of suitable polymer is 1.5kD - 250kD. We have used commercial preparations of 3, 6, 9, 12, 18, 22, 60, 65 & 90kD. Hydrolysis rate of the polymer is related to the molecular weight and thus the lower molecular weight polymers will degrade more quickly.

When microparticles are honey-combed in structure, as has been proposed in Sah *et al*, the aqueous phases in the polymer matrix will not be lyophilised during freeze drying. This could explain the frequently observed denaturation of the biological agent and decreasing pH of the prior art preparations as the wet polymer hydrolysed to glycolic and lactic acids. We have observed that microparticles made according to the present invention collapse if treated harshly suggesting they have empty interiors from which water has been removed.

The microparticles of the present invention are in fact shell like, (Fig 3b) and not solid, honey-combed or enclosing a material phase of any sort other than water when hydrated. Further, the microparticles of the present invention can be dried, for example by freeze drying without loss of the spherical structure (Fig 4a). The present invention thus enables preparation of microparticles containing dry pDNA which will be far more stable than aqueous preparations.

Manufacture of the microparticles of the invention enables incorporation into the microparticles of any water-soluble substances, even any water-dispersible substances, though preferably the bioactive agent is selected from RNA, protein antigen, non-protein antigen, protein-or peptide-conjugated polysaccharides, protein-or peptide-DNA complexes, synthetic peptides, synthetic proteins, DNA viruses, cytokines, cancer therapeutics, mini-genes and water-soluble pharmaceuticals. The bioactive agent may further be accompanied by excipient and/or adjuvant, one example of which is alum.

Virtually any recombinant or native protein antigen may be encapsulated using

- 9 -

the methods of the present invention, in particular any recombinant or native antigen useful to immunise against any pathogenic organism. By way of example only, a protein antigen for incorporation into microparticles of the present invention is optionally selected from:-

- 5 (a) the antigens FHA, PT, 69kD-Pertactin, tetanus toxin, gp48, NS1, Capsid, gp350, NS3, SA, I, NP E, M, gp340, F, H, HN, 35kD protein, BP1, E1, E2, C, M, E and MSHA according to table 4; and
- (b) immunogenic fragments, variants and derivatives of the
10 polypeptides of (a).

Details of Accession numbers of gene sequences for these antigens are listed in table 4.

- 15 In a (water-in-oil)-in-water emulsion system of the present invention the water droplet containing the DNA or bioactive agent is dispersed within the oil which is itself dispersed within the second water phase. This might result in the formation of 'currant buns' the currants being the aqueous phase + DNA and the bun the polymer. In the present invention, the generation of this initial
20 water-in-oil emulsion and its dispersion into the second water phase play a major part in the formation and character of the final microparticle. The generation of our microparticles is heavily influenced by the initial emulsification stage as the resultant microparticles correspond to individual droplets from the dispersed DNA- or bioactive agent- containing aqueous phase. It is around these
25 that polymer has condensed. In preferred embodiments of the invention, the concentration of the polymer and the ratio of polymer solution to the initial aqueous phase are controlled to influence the microparticles obtained.

- Specifically, in preferred embodiments of the invention the ratio of
30 solvent:aqueous phase in formation of the water-in-oil emulsion is in the range of 4:1 to 20:1 (volume :volume), more preferably 5:1 to 15:1 and even more preferably 5:1 to 12:1 (volume:volume). Selection of ratios within these ranges

- 10 -

dictates the formation of an initial water-in-oil emulsion that is instrumental to determining the size range and architecture of the resultant microparticles, and improves the efficiency of incorporation of DNA into the microparticles. When the ratio of solvent:aqueous phase falls below about 4:1, there is a significant reduction in the incorporation of bioactive agent, such as DNA, into the microparticles. This is the result when insufficient polymer containing phase is available to effectively coat the aqueous droplets thus preventing shell formation and loss of bio-active agent into the aqueous environment of the second emulsion phase. The incorporation efficiency increases with a ratio of at least 4:1 and preferably at least 5:1. When the ratio goes beyond about 20:1, then it becomes extremely difficult to prevent formation of "currant bun"-type structures as shown in the Sah paper. This is a consequence of an excess of polymer containing phase, which percents the dissociation of individual shell structures resulting in conglomerations into "currant bun" structure and solid microparticles showing polymer matrix inside. The structures are many times larger than 10 microns in diameter and thoroughly unsuitable for oral immunisation according to the invention. Below about 20:1 and preferably below about 15:1, the propensity for the method to produce these "currant bun"-type structures falls and microparticles of up to 10 microns in diameter are more readily formed.

Solvents suitable for use in dissolving the polymer are preferably of low toxicity and are ideally selected from the category III list of solvents approved for pharmaceutical use. Solvents in the category II list can also be used, though for human use it will usually be necessary to determine the amount of residual solvent in the particles before such particles can be pharmaceutically approved. Such solvents will dissolve preferably at least around 50mg/ml of polymer and should be substantially immiscible with water, thereby facilitating the formation of a (water-in-oil)-in-water emulsion. In preferred embodiments of the invention the solvent exhibits a high vapour pressure so that the final solvent extraction step can be carried out quickly. Examples of solvents suitable for use in the method of the invention include ethyl acetate, dichloromethane, chloroform,

- 11 -

propylene carbonate, and mixtures thereof. Acetone can also be used in solvent mixtures, ie as a cosolvent, but not on its own as it is miscible with water.

It is further preferred that the solvent comprises ethyl acetate either with or
5 without a co-solvent which increases the amount of polymer that can be dissolved in the solvent. A number of co-solvent combinations are suitable, provided that the major proportion of the mixture is ethyl acetate (or another pharmaceutically accepted solvent). The advantages for employing co-solvent systems are twofold. First, the solvent combinations dissolve a greater weight
10 of polymer than in single component solvents, which is advantageous when attempting to reduce the ratio of solvent aqueous phase in generation of the initial emulsion. Second, the co-solvents facilitate the dissolution of high molecular weight polymers, allowing the maintenance of critical solvent aqueous phase ratios with the less soluble polymers. Examples of such co-solvents
15 include propylene carbonate and acetone. More generally, the invention also provides use of a co-solvent in combination with any suitable solvent for preparation of a polymer solution for use in preparation of a microparticle, and the methods of the invention are thus not limited to solvents which necessarily comprise ethyl acetate. For example, a solvent being a combination of
20 dichloromethane and acetone, dissolving a greater weight of polymer than is dissolved by dichloromethane on its own, is another aspect of the invention. Further aspects of the invention include the use of any pharmaceutically solvent in combination with a co-solvent such as acetone whereby the combined solvent dissolves a greater weight of polymer than the pharmaceutically
25 acceptable solvent on its own.

It is also preferred especially when DNA is being encapsulated that the emulsification steps of the method be carried out under conditions of reduced shear stress, and this is optionally achieved by use of an emulsifying energy,
30 such as speed in the case of an emulsifying mixer, that is sufficient to obtain an emulsion and to form microparticles in the desired size range but not so high that all DNA is damaged by excessive shear. In an embodiment of the

- 12 -

invention, described below, the emulsifying mixer speed is modified so that at least 25% of the DNA biological activity (assayed by transformation of competent bacteria or transfection of cultured cells) is retained in the resultant microparticles that contain DNA. Suitable mixer speeds in the case of a
5 Silverson mixer are below 8000 rpm, preferably below 6000 rpm, and in specific embodiments described below the speeds are about 3000 rpm or above about 2000 rpm. Every variation of equipment that can be used to generate the emulsion phases will require assessing at different speeds and under different conditions. It is left for those skilled in the art to identify optimal speeds and
10 duration of emulsification for each piece of apparatus. Using the Silverson Homogeniser at speed above 8000 rpm, there is significant damage to DNA and below about 2000 rpm there is very little or no formation of microparticles of size 10 microns or less in diameter. A more detailed analysis of the effect of mixer speed using a Silverson mixer is found in WO-A-97/17063.

15 The steps preliminary to and during formation of microparticles are thus adapted to input sufficient energy so as to form microparticles in the desired size range, which is typically 0.01-10 microns, but not so much energy that DNA or bioactive agent is damaged during the process. There is a balance required as
20 more vigorous agitation such as through higher mixer speeds typically results in smaller microparticle sizes. But, DNA may be damaged by excessive agitation during emulsification. On the other hand, reducing the energy input during emulsion formation may have the effect that no emulsion is formed and no microparticles can be obtained. The invention enables a balance of these
25 competing factors, to provide for formation of microparticles retaining an acceptable degree of biological activity.

In further embodiments of the invention, the method comprises maintaining the emulsification of the polymer and aqueous solutions to form the initial water-in-
30 oil emulsion over a prolonged period of time, so as to obtain aqueous phase droplets a high proportion of which will result in microparticles of a size up to 10 microns in diameter. This has the advantage that with prolonged agitation

- 13 -

during formation of the water-in-oil emulsion the size distribution of the microparticles becomes more homogenous.

Longer emulsification reduces the proportion of microparticles that are bigger
5 than 10 microns. In addition, prolonged emulsification at different
emulsification energies produces size distributions centred around different
points - higher energies producing smaller average microparticle sizes. Hence,
the method can be modified so as to tailor the obtained sizes to the size
desired. Some prior art has suggested in connection with uptake of
10 microparticles through the Peyer's patches that microparticles of 1 to 5 microns
and those of 5 to 10 microns are taken up differently, and the present invention
facilitates manufacture of microparticles with any chosen average diameter. It
is also preferred to maintain the agitation of the polymer and aqueous solutions
to form the water-in-oil emulsion over a prolonged period of time so as to obtain
15 microparticles the majority of which and preferably substantially all of which are
of a size up to 10 microns in diameter and more than 0.1 microns in diameter.
In this context, reference to substantially all particles is intended to indicate that
at least three quarters of the particles by number, and preferably at least 90%,
are of size within the chosen diameters.

20

It is also preferred to intercalate rest pauses between emulsion cycles so as
to allow any heat build up that occurs during agitation of the emulsion to
dissipate and thereby reduce damage to the bioactive agent being encapsulated.

25 In particular embodiments of the invention, described below in further detail,
there is provided a method for encapsulating a bioactive agent in a polymer
microparticle, comprising:-

dissolving the polymer in a solvent to form a polymer solution;
30 preparing an aqueous solution of a bioactive agent;
combining the polymer and aqueous solutions with agitation to form a
water-in-oil emulsion;

- 14 -

adding the water-in-oil emulsion to a further aqueous phase with agitation to form a (water-in-oil)-in-water emulsion;

adding the (water-in-oil)-in-water emulsion to excess of an aqueous phase to extract the solvent, thereby forming polymer microparticles of a size up to 10 microns in diameter;

wherein agitation of the polymer and aqueous solutions, and agitation of the water-in-oil emulsion and the further aqueous phase, are carried in a blender.

10 The invention further provides for a method of preparing bioactive agent encapsulated within polymer microparticles of the invention using a blending apparatus for performing the emulsion steps. The blending apparatus comprises:

a container for a liquid to be mixed;

a blade for mixing liquid in the container;

wherein the blade rotates about an axis of rotation and exerts a mixing action that includes driving liquid in a direction substantially along the axis of rotation.

The rotational action of the blade imparts emulsion energy into the liquid by establishing a rapidly circulating vortex within the liquid of the chamber. Thus the action of the apparatus is distinguished from other mixers which employ spinning probes that draw up liquid which is then extruded through an emulsor screen. Such an action produces significant shear forces and is highly damaging to DNA. An advantage of the present apparatus is that the bioactive agent such as DNA is subjected to lower shear and other stresses during encapsulation. The blending action of the apparatus is also distinguished from sonication mixers which employ ultrasound pulses to impart emulsion energy in to the liquid. Ultrasound is also damaging to DNA and can cause fragmentation of polynucleotide strands.

- 15 -

The emulsion steps are preferably carried out using apparatus which is a blender/mixer of the Waring (RTM) type. Thus the blender comprises a chamber for blending and mixing the emulsion, and further comprises a rotatable blade fixably mounted within said chamber, which blade includes a plurality of blade
5 end tips extending upwardly and/or downwardly from the blade and may be roughly at 30-60 degrees to the blade or even perpendicular to the blade and thus parallel to the axis of rotation.

It is an advantage of use of the apparatus according to the invention that this
10 method is appropriate for scale-up to industrial production of microparticles, in sharp contrast to the use of a homogeniser which would be appropriate only for tiny volumes of liquids suitable for experimental purposes.

The present invention also relates to modification of the polymer shell to
15 achieve modified release rates of the particle contents.

Accordingly, the present invention provides a composition comprising microparticles of less than 10 microns in diameter which contain an external polymer shell and an internal aqueous solution of a bioactive agent, said
20 composition comprising a microparticle wherein the polymer is PLG of molecular weight less than 50kD, and preferably down to 1.5kD.

The present invention additionally provides a composition comprising microparticles of less than 10 microns in diameter which contain an external
25 polymer shell and an internal aqueous solution of a bioactive agent, said composition comprising a microparticle wherein the polymer is PLG of molecular weight more than 70kD, and preferably up to 250kD.

The present invention still further provides a composition comprising mixtures
30 of microparticles of less than 10 microns in diameter which contain an external polymer shell and an internal aqueous solution of a bioactive agent, said composition comprising a first microparticle wherein the polymer is PLG of

molecular weight less than 50kD and a second microparticle wherein the polymer is PLG of molecular weight more than 70kD. Very complex mixtures of individual types of polymer particles can be mixed and used as combination vaccines or therapies.

5

Where possible, however, the molecular mass distributions of the polymers suitable for use in the invention are quoted in terms of their inherent viscosities (i.v.). The i.v. is a quantifiable value that is easily measured and is known to be correlated to molecular mass distribution. It is therefore often a more accurate
10 description of the molecular mass distribution of a polymer than an "average molecular weight" value. The i.v. is measured as a function of volume per unit mass and is generally expressed in decilitres per gram (dl/g).

In one aspect of the invention there is provided a polymer microparticle, of size
15 up to 10 microns in diameter, and containing a bioactive agent, wherein the polymer is PLG of inherent viscosity less than 0.5 dl/g. The bioactive agents can be DNA, such as DNA encoding a polypeptide than is expressed following administration to a recipient or encoding an antisense sequence.

20 This confers the advantage that the microparticle can be broken down once inside a recipient and having been taken up by antigen presenting cells but before a significant proportion of the DNA has degraded.

It is preferable to obtain a release profile where the maximum amount of
25 encapsulated DNA is released when the majority of the microparticles have been taken up and internalised by cells. Typical release profiles of preferred embodiments of the invention provide for 30 to 40% release of the encapsulated DNA between 1 and 20 days, more preferably between 2 and 10 days, following administration to an animal. When administration is via the oral
30 route it is preferred that significant DNA release occurs after passage of the microparticles through the stomach and into the gut of the animal where the microparticles are then taken up by the M cells of the Peyer's patches where

- 17 -

they then degrade and release the encapsulated DNA.

It has been discussed above how using PLG of low i.v. (typically low molecular weight) might give improved immunisation, due to more rapid breakdown of particles and release of particle contents than using the standard 60kD PLG (having high i.v.). Nevertheless, if a composition comprising microparticles having a range of i.v.s (and hence a range of molecular weights) is used, it can be an advantage that this combination produces an advantageous immunisation effect.

10

In a specific embodiment of the present invention release profiles for the microparticles can be engineered by adjusting the i.v. and/or molecular mass distribution of the polymer. In a specific example of the invention described in more detail below, this adjustment is made by combining PLG preparations of higher and lower i.v. values to produce a composite polymer of intermediate i.v. The microparticles made with intermediate i.v. PLG exhibit a modified DNA release profile wherein the greater the proportion of the lower i.v. polymer present the more quickly the DNA is released. In this way microparticles of the invention are produced that exhibit the desired release profiles suitable for oral and other administration routes.

20

Preferably, the i.v. is in the range of 0.1 to 0.4 dl/g. In specific embodiments of the invention it has been found that microparticles with polymer shells made of PLG having an i.v. within this range have exhibited good DNA release profiles and retained good particulate structure when examined under the scanning electron microscope. In particular examples of the invention described below, particles have been made with mixtures of PLG having i.v. of 0.19dl/g and 0.39dl/g and these particles showed very good release profiles over time.

25

The present invention relates also to vaccination therapies and methods using the microparticles of the present invention. Accordingly, the present invention further provides a method of making a microparticle for use as or in a

30

- 18 -

vaccinating composition, comprising following the methods of the present invention, wherein the microparticle contains DNA coding for an antigen.

Still further the present invention provides a composition for use as or in a
5 vaccinating composition, comprising a composition of the invention as previously recited wherein the microparticle contains DNA coding for an antigen, and use of a composition according to the invention in manufacture of a medicament for vaccination, wherein the microparticle contains DNA coding for an immunogen.

10

In a specific embodiment of the present invention, the microparticles are located within or are surrounded by a matrix. Such a matrix is made preferably from biodegradable material that breaks down over time to release microparticles either in a steady stream or in periodic pulses. The depot of the invention is
15 typically administered subcutaneously or implanted into another part of the body. It is preferable that the depot be partly resistant to rehydration so that the particles located within the core remain in a dehydrated state until they are released, thereby ensuring that the encapsulated DNA is of high integrity for substantially the full lifetime of the depot. Examples of materials suitable for
20 manufacture of such depots are commonly known to the art and include but are not limited to, polyhydroxybutyric acid; polycaprolactone; silicone; ethylene vinyl acetate and polyvinyl acetate.

Following the methods of preferred embodiments of the invention, the majority
25 (typically 90% or more) of microparticles obtained lie in the size range of 10 or less micrometres. Electron micrographs of microparticle preparations show the majority of these seem to be shells (Fig 3b). The presence of smaller microparticles is not detrimental to the efficacy of the preparation as microparticles smaller than 1 micrometres are still known to be actively
30 phagocytosed by M cells and subsequently transferred to cells of the gut associated lymphoid tissue (GALT). Indeed, small (less than 2 microns diameter) sized microparticles have been suggested as being advantageous in

- 19 -

promoting uptake by phagocytic cell and M-cells of the gut. The major barrier to preparing small particles containing active bio-active molecules has been the need to introduce large amounts of potentially damaging energy in order to prepare the desired size distribution. A clear advantage of the current invention is that the additional control of energy input using the blender technology will allow the generation of smaller particles without denaturation of the bio-active molecule. It also offers the possibility of large-scale production of these potentially improved delivery vehicles.

Size of microparticles is very important in the process of phagocytosis of microparticles by M cells and for cell to cell transfer. It is known that microparticles of sizes greater than 10 microns are not taken up and transported across the gut epithelium. In some of the preparations made according to the prior art we have seen significant numbers of microparticles with diameters 10 to 60 microns, ie larger than the optimum size required. If it is considered that the volume of a sphere is calculated on a cube function, it is entirely feasible that in such preparations most of the pDNA, could be contained inside the large shells. This would make it unavailable to the immune system and thus the dose could be considerably lower than anticipated, a problem overcome herein as following the methods described in the present invention which results in microparticle preparations having a majority of microparticles in the desired size range of up to 10 microns.

Control of the release characteristics of the microparticles of the invention has been addressed. Exactly how the bioactive agent is released from the micro-shell is unclear - possibly as a burst once the shell degrades by hydrolysis. This results in a sustained release as microparticle disruption occurs at different times. The time range may conceivably be over a shorter period than previously contemplated and is controllable through polymer molecular weight as described.

A range of surfactants are suitable for use in the method of the invention, and

- 20 -

the present invention is not limited to the particular surfactant used in the examples, polyvinylalcohol. Other acceptable surfactants are known in the art, and methyl cellulose is a further example. The surfactant has the role of stabilising the double emulsion. Choice of aqueous surfactant is a matter for the skilled person and this choice may be made with regard to the choice of polymer and polymer solvent.

Other relevant background to the uptake of microparticles by M cells in the gut is provided in:-

10

Jepson MA *et al*, Journal of Drug Targeting, 1993, Vol. 1, pp 245-249;
Howard KA *et al*, Pharmaceutical Science Communications, 1994, Vol. 4, pp 207-216;

Neutra MR *et al*, Cell, 1996, Vol. 86, pp 345-348;

15

Florence AT, Pharmaceutical Research, 1997. Vol. 14, pp 259 *et seq*;
O'Hagan DT, J. Anat., 1996, Vol. 189, pp 477-482; and
Kreuter J, J. Anat., 1996, Vol. 189, pp 503-505.

The present invention is now described in specific embodiments with reference to the accompanying drawings in which:-

20

Fig. 1 is an agarose gel that shows the physical state of the DNA recovered from microparticles prepared with dichloromethane (prior art solvent) and ethyl acetate (in accordance with the invention);

25

Fig. 2 shows release profiles of DNA from PLG microparticles prepared from high or low molecular weight polymer; and

Figs 3a, 3b, 4a and 4b show scanning electron micrographs of prior art and inventive microparticles.

Fig. 5 shows DNA release profiles of preparations of microparticles made from PLG of different inherent viscosities.

30

Fig. 1 shows an agarose gel showing the relative amounts and the relative

- 21 -

physical state of DNA recovered from equivalent weights of PLG microparticles prepared using either dichloromethane (prior art: Lane 3) or ethyl acetate (in accordance with the invention: Lanes 4 (high mol. wt. PLG), Lane 5 (low mol. wt. PLG)). Lane 2 is the control DNA (stock) showing a large proportion of super-coiled form (lower band) and Lane 1&6 are the molecular weight markers. Comparison of Lane 3 with Lanes 4&5 suggests that ethyl acetate not only promotes the encapsulation of DNA, but also results in the retention of a greater proportion of the super-coiled form (lower band) compared with open circle form (upper band). It should be noted that the lower incorporation of DNA in the preparation analyzed in Lane 4 (high mol. wt. PLG) is a result of microparticle formation in the presence of limiting polymer due to its lower solubility in ethyl acetate.

Fig. 2 shows the *in vitro* release kinetics of the PLG microparticles, of the invention. Microparticles were prepared using high (■-■), or low (◇-◇) molecular weight PLG. DNA concentrations in aliquots of supernatants serviced from sterile incubations of microparticle suspended in buffer maintained at 37 degrees C, were measured using optical absorption spectroscopy.

Fig. 3a shows a preparation of PLG microparticles (prepared according to the present invention) that have been applied to an EM specimen grid as a lyophilised powder. The uniformity of spherical structure indicates that the particles are not adversely effected by freeze-drying.

Fig. 3b demonstrates the "shell-like" structure of PLG microparticles (according to the present invention) prepared under optimal conditions. The specimen was freeze fractured. The hollow interiors of these particles can be clearly seen and are very different from examples given in the prior art.

Fig. 4a shows a preparation of PLG microparticles (prepared according to the present invention), that have been dried from a hydrated slurry onto EM specimen grid at room temperature. The stresses encountered as the water

evaporated have induced structural collapse to differing extents, indicating that the microparticles may comprise a "shell-like" structure with a hollow interior.

Fig. 4b shows the internal structure of microparticles prepared under conditions of excess (greater than 4:1) solvent: aqueous ratio. Preparations were frozen using liquid nitrogen and specimens were fractured whilst frozen to reveal particles with different elaborate internal structures.

Fig. 5 shows the percentage of DNA released over time from PLG microparticles made from PLG preparations of varying inherent viscosities. Low inherent viscosity PLG is denoted RG502 and higher inherent viscosity PLG is denoted RG503. DNA release from microparticles made from mixtures of 50:50 and 75:25 of RG502 and RG503 are also shown and represent PLG preparations of inherent viscosity in between those of RG502 and RG503.

Example 1

Microencapsulation standard method

Microparticles were prepared following the method now set out, which method is referred to as the microencapsulation standard method.

1. A 10% (w/v) 50:50 low iv (60kD) PLG solution in ethyl acetate (dried over sodium carbonate) is prepared by heating the mixture to around 37 degrees C and rolling until dissolution is complete.
2. A plasmid DNA solution is prepared to a concentration of 10mg/ml in STE buffer (10mM Tris HCl, pH 8.0; 1mM EDTA; 150mM NaCl).
3. The PLG ethyl acetate solution (4ml) is homogenised at 3000 rpm using a Silverson homogeniser and the plasmid DNA (600 microlitres) added. The resultant mixture is homogenised for a further 2.5 minutes.
4. An 8% (w/v) aqueous polyvinyl alcohol solution (92ml) is homogenised

- 23 -

in a round bottom flask at around 3000 rpm the first emulsion is added and the resultant mixture homogenised for a further 2.5 minutes.

5 5. The emulsion is poured into doubly distilled water (100ml-1L) at 37 degrees C and stirred for at least 20 minutes.

6. The microparticles are recovered by centrifuging at 10,000rpm for 25 minutes at 25 degrees C.

10 7. The microparticles are washed by resuspending in doubly distilled water and centrifuging. This wash step is repeated such that a total of 5 spins are performed, to facilitate removal of the solvent and the emulsion stabilizer.

15 8. Following the 5th spin the pelleted microparticles are recovered as a slurry in a minimal quantity of doubly distilled water and freeze dried. A typical preparation is shown in Fig. 3a.

Variations to the standard method

20 Based on the above standard method a number of variations were made to the steps and/or the components, with results as follows:-

1. Solutions of both 50:50 low (50kD and below) MW and 50:50 high (70kD and above) MW PLG in ethyl acetate have been studied and found to produce microparticle preparations of size up to 10 microns. Other PLG
25 compositions e.g. 75:25 and polymers with higher or lower MW are also suitable for the microparticles and methods of the invention.

2. The w/v ratio of PLG to EA has been studied. With the high MW. polymer a reduction to 0.04 g/ml led to a marked decrease in incorporation efficiency
30 (Fig.1). The maximum amount tested was 0.125 g/ml, which improved encapsulation, produced microparticles in the size range 1 to 10 microns, but resulted in turbid solutions prior to encapsulation.

- 24 -

3. The use of co-solvents to increase the concentration of PLG in the organic phase has been studied. Co-solvents such as propylene carbonate and acetone in combination with ethyl acetate have enabled preparation of an initial water-in-oil emulsion having an increased amount of polymer dissolved in the oil (solvent) phase. This advantageously has been found to improve efficiency of incorporation of DNA into microparticles.

4. The ratio of aqueous phase to polymer phase in the first emulsification step has been varied within the ranges specified, and it is found that honey-comb structures are avoided and microparticle size can be controlled giving preparation of particles substantially all within the desired range and with the desired structures.

5. Homogenisation speed for first emulsification has been varied. Using a Silverson mixer, it is possible to increase the homogenisation speed to 4000 rpm while still retaining some DNA viability. However, 2000 rpm gives good DNA viability and is sufficiently non-damaging for extended homogenisation at this speed not to lead to significant DNA damage whilst allowing formation of microparticles having a narrow size distribution. 3000 rpm can also be used for extended time periods without significant DNA damage.

6. The concentration of PVA used is thought to be in excess and concentrations of 2% have been suggested as adequate. This will also have an effect on the viscosity of the mixture which may also affect microparticle stability and size.

7. The lowest volume of water successfully used in the final stage has been 150ml. The stirring has been carried out at room temperature using water initially at 37 degrees C, and stirring has been continued for times from 20 minutes up to 1.5 hours, though there seems to be no theoretical upper limit to the stirring time.

- 25 -

8. The temperature at which the procedure is carried out will effect parameters such as the viscosity of the solutions and solubility of reagents in other phases. A single experiment carried out at 4 degrees C with ethyl acetate resulted in lower incorporation.

5

Example 2

Comparison of Solvents and High and Low Molecular Weight 50:50 PLG

The following experiments were carried out using the standard method, in which EA = ethyl acetate and DCM = dichloromethane. The PLG volume in each case was 4 ml. No EtOH was used in any of the experiments. The results are shown in Table 1.

10

Table 1

PLG M.Wt.	Concentration (%)	Solvent	Incorporation (ug/mg)	Efficiency (%)
60kD	12.5	DCM	1.4	8.9
90kD	10.0	DCM	1.4	6.6
60kD	12.5	EA	18.3	74.3
90kD	10.0	EA	11.8	62.8

15

20

Example 3

Physical state of DNA recovered from the microparticles

Figure 1 - shows the physical state of the DNA recovered from microparticles prepared with dichloromethane and ethyl acetate. The lowest band is supercoiled DNA. The greater the intensity of this band the more the integrity of the DNA has been retained.

25

Example 4

Transfection results

Experiments were carried out with Vero cells. DNA was recovered from

30

- 26 -

microparticles and Vero cells transfected using SUPERFECT REAGENT (Registered Trade Mark).

Further experiments were carried out transfecting vero cells with microparticles containing DNA but using no additional reagents to enhance transfection. The extent of transfection was assessed using a commercially available luciferase assay system and the results are shown in Table 2.

Table 2

Sample	Reading
Negative control (cells only)	9
Positive control (2ug pLuc)	100000 (diluted 1:100)
DNA recovered from EA mps (2ug pLuc)	40748 (diluted 1:100)
pLuc containing mps prepared with EA	29937

Example 5

Effect of PLG concentration

Table 3 below shows the results obtained when the concentration of low mol.wt (60kD) PLG was reduced. In these comparative experiments ethyl acetate was used as solvent, the volume of the organic phase was kept at 4ml. Homogenisation speeds of 3000 rpm were used in all cases.

Table 3

PLG Concentration (%)	Incorporation (ug/mg)	Efficiency (%)
10.0	18.3	74.3
8.0	12.4	51.1
6.0	12.3	39.1

Example 6**Use of co-solvents.**

High molecular weight PLG is not totally soluble at or above 10% w/v in ethyl acetate. In order to increase the PLG concentration the application of mixtures of solvents such as ethyl acetate and propylene carbonate was investigated.

80% ethyl acetate : 20% propylene carbonate gives a clear solution of 10%w/v PLG.

Example 7**Release kinetics**

Samples of microparticles prepared with ethyl acetate using high and low MW polymers were studied to determine the release characteristics. The microparticles were added to sterile STE buffer and maintained at 37 degrees C with an appropriate antibacterial agent. Samples were removed at intervals and the DNA released and retained determined. From this data percentage release with time profiles were determined. The results are shown below in Figure 2.

Example 8**Microparticles prepared using polycaprolactone as th polymer**

Microparticles are prepared using polycaprolactone polymer by the following

- 28 -

method

1. A 100mg/ml solution of polycaprolactone (average MW = 14 kD; Aldrich Chemical Company) in ethyl acetate is prepared by vortexing until dissolution is complete.
5
2. A plasmid DNA solution is prepared to a concentration of 9.9mg/ml in STE buffer (10mM Tris HCl, pH 8.0; 1mM EDTA; 100mM NaCl).
- 10 3. The polycaprolactone ethyl acetate solution (4ml) is homogenised at low power in a Waring blender and the plasmid DNA (400 microlitres) added. The resultant mixture is homogenised for four fifteen second cycles intercalated with 15 second rest periods.
- 15 4. An 8% (w/v) aqueous polyvinyl alcohol solution (66ml) is homogenised in a Waring blender at low power, the first emulsion is added and the resultant mixture homogenised for four fifteen second cycles intercalated with 15 second rest periods.
- 20 5. The emulsion is poured into doubly distilled water (300ml) at 37 degrees C and stirred for at least 20 minutes.
6. The microparticles are recovered by centrifuging at 10,000rpm for 25 minutes at 25 degrees C.
- 25 7. The microparticles are washed by resuspending in doubly distilled water and centrifuging. This wash step is repeated such that a total of 5 spins are performed, to facilitate removal of the solvent and the emulsion stabilizer.
- 30 8. Following the 5th spin the pelleted microparticles are recovered as a slurry in a minimal quantity of doubly distilled water. Microparticles are obtained predominantly in the size range 1-10 microns in diameter and are then freeze

- 29 -

dried for storage.

Example 9

Modification of release profiles of PLG microparticles by altering the inherent viscosity of the polymer

The procedure of Example 8 was followed using the following PLG preparations:-

- (a) inherent viscosity of 0.19 dl/g (RG502, from Boehringer Ingelheim)
- (b) inherent viscosity of 0.39 dl/g (RG503, from Boehringer Ingelheim)
- (c) mixture of 50:50 by weight of PLG of inherent viscosities 0.19 and 0.39 dl/g (mixture of RG502 and RG503).
- (d) mixture of 75:25 by weight of PLG of inherent viscosities 0.19 and 0.39 dl/g (mixture of RG502 and RG503).

Microparticles of size predominantly 1 to 10 microns are obtained and the profiles of release of DNA from the particles measured and illustrated in figure 5.

Example 10

The protocol of Example 8 was followed with the following variations: 8ml of polymer solution and 0.8ml of plasmid solution were used and 132ml PVA was used to stabilize the first emulsion.

Plasmids were encapsulated in PLG as follows:

- (a) mixture of 50:50 RG502:RG503;
- (b) the (a) mixture repeated;
- (c) mixture of 70:30 RG502:RG503;
- (d) the (c) mixture repeated.

Microparticles of size predominantly 1 to 10 microns diameter were obtained

- 30 -

and tested for efficiency of DNA incorporation. From the microparticles of (a) and (b) 65% was recovered as encapsulated DNA and from those of (c) and (d) 69%. This % figure does not make allowance for the fact that a proportion of the DNA is lost in isolation of the particles, for example by centrifugation, and
5 that as a consequence recovery can not be 100% efficient. The figures achieved in these examples thus represent very high efficiency of incorporation.

The invention thus provides further and improved methods of encapsulation of bioactive agents, DNA in particular, and further an improved compositions
10 comprising microparticles containing bioactive agents, for gene therapy and/or vaccination and other applications.

Table 4

**ACCESSION NUMBERS OF GENE SEQUENCES IDENTIFIED AS PUTATIVE OR
PROTECTIVE ANTIGENS AGAINST SPECIFIC PATHOGENS**

5

10

15

20

Organism	Protein/Glycoprotein	Accession Number	Database
Bordetella Pertussis	FHA; PT	P12255; M13223	Swiss Prot; Genbank
Bordetella Bronchiseptica	68kD-Pertactin	X54815	Genbank
Clostridium Tetani	Tetanus Toxin	P04958	Genbank
CMV	gp48	A32390	Swiss Prot
Dengue Virus	NS1; Capsid	S37468; Z794047	Swiss Prot
EBV	gp350	A43042; S33008; A03762	Swiss Prot
Flavivirus	NS3	S79821; S79825; S79826; S79830	
Hepatitis B Virus	SA	V00867; X02763	Genbank
Herpes Simplex Virus	I	P06487	Genbank
Influenza Virus	NP	H36754	Swiss Prot
JEV	E; M; gp340	M18370	Swiss Prot

- 32 -

Measles Virus	F;H	D00090; N00090; Z80790	Genbank
Mumps Virus	HN	X93178	Genbank
Mycobacteria Tuberculosis	35kD protein	M69187	Genbank
Rotavirus	VP1	P35942	Swiss Prot
Rubella Virus	E1;E2	A27505; D00156	Swiss Prot, Genbank
TBE	C;M;E	X07755; M97369	Genbank
Vibrio Cholerae	MSHA	X77217	Genbank

- 33 -

CLAIMS:

1. A method for encapsulating a bioactive agent in a polymer microparticle, comprising:

dissolving polymer in a solvent to form a polymer solution;

preparing an aqueous solution of a bioactive agent;
combining the polymer and aqueous solutions with agitation to form a water-in-oil emulsion;

adding the water-in-oil emulsion to a further aqueous phase with agitation to form a (water-in-oil)-in-water emulsion;

adding the (water-in-oil)-in-water emulsion to excess of an aqueous phase to extract the solvent, thereby forming polymer microparticles of a size up to 10 microns in diameter, said microparticles containing bioactive agent in aqueous solution;

wherein the solvent comprises ethyl acetate.
2. A method according to claim 1, wherein the ratio of solvent:aqueous phase in formation of the water-in-oil emulsion is in the range of 4:1 to 20:1 (volume:volume).
3. A method according to Claim 2 wherein the ratio of solvent:aqueous phase in formation of the water-in-oil emulsion is in the range of 5:1 to 12:1 (volume:volume).
4. A method according to any of claims 1 to 3 wherein the polymer is PLG and the solvent comprises ethyl acetate and a co-solvent which increases the amount of PLG that can be dissolved in the solvent.

- 34 -

5. A method according to any of claims 1 to 4 comprising maintaining the agitation of the polymer and aqueous solutions to form the water-in-oil emulsion over a prolonged period of time so as to obtain microparticles the majority of which are of a size up to 10 microns in diameter.
6. A method according to any of claims 1 to 5 comprising maintaining the agitation of the polymer and aqueous solutions to form the water-in-oil emulsion over a prolonged period of time so as to obtain microparticles the majority of which are of a size up to 10 microns in diameter and more than 1 micron in diameter.
7. A method according to any of claims 1 to 6 wherein the polymer is PLG of a molecular weight greater than 70kD.
8. A method according to any of claims 1 to 6 wherein the polymer is PLG of a molecular weight less than 50kD.
9. A method according to any of Claims 1 to 8 wherein the bioactive agent is selected from RNA, protein antigen, non-protein antigen, protein-or peptide-conjugated polysacharrides, protein-for peptide-DNA complexes, synthetic peptides, synthetic proteins, DNA viruses, cytokines, cancer therapeutics, mini-genes and water-soluble pharmaceuticals, optionally accompanied by excipient and/or adjuvant, one example of which is alum.
10. A method of encapsulating DNA in a polymer microparticle, comprising:
dissolving polymer in a solvent to form a polymer solution;
preparing an aqueous solution of DNA;
combining the polymer and DNA solutions with agitation to form a water-in-oil emulsion;
adding the water-in-oil emulsion to a further aqueous phase with agitation to form a (water-in-oil)-in-water emulsion;

adding the (water-in-oil)-in-water emulsion to excess of an aqueous phase to extract the solvent, thereby forming polymer microparticles of a size up to 10 microns in diameter, said microparticles containing DNA in aqueous solution;

wherein the DNA comprises a coding sequence and induces expression of the coding sequence in a recipient following oral administration, and wherein the solvent comprises ethyl acetate.

11. A method according to claim 10, wherein the ratio of solvent:aqueous phase in formation of the water-in-oil emulsion is in the range of 4:1 to 20:1 (volume:volume).
12. A method according to claim 11 wherein the ratio of solvent:aqueous phase in formation of the water-in-oil emulsion is in the range of 5:1 to 12:1 (volume:volume).
13. A method according to any of claims 10 to 12 wherein the solvent comprises ethyl acetate and a co-solvent which increases the amount of polymer that can be dissolved in the solvent.
14. A method according to claim 13 wherein the polymer is poly-(DL lactide-co-glycolide) (PLG) and the concentration of PLG in the polymer solution is at least 10% w/v.
15. A method according to any of claims 10 to 14, comprising maintaining the agitation of the polymer and aqueous solutions to form the water-in-oil emulsion over a prolonged period of time so as to obtain microparticles the majority of which are of a size up to 10 microns in diameter.
16. A method according to any of Claims 10 to 15 comprising maintaining the agitation of the polymer and aqueous solutions to form the water-in-

- 36 -

oil emulsion over a prolonged period of time so as to obtain microparticles the majority of which are of a size up to 10 microns in diameter and more than 0.1 microns in diameter.

17. A method according to any of claims 10 to 16 wherein the polymer is PLG of a molecular weight greater than 70kD.
18. A method according to any of claims 10 to 16 wherein the polymer is PLG of a molecular weight less than 50kD.
19. A composition comprising microparticles of less than 10 microns in diameter which contain an external polymer shell and an internal aqueous solution of a bioactive agent, said composition comprising a microparticle wherein the polymer is PLG of molecular weight more than 80kD.
20. A composition comprising microparticles of less than 10 microns in diameter which contain an external polymer shell and an internal aqueous solution of a bioactive agent, said composition comprising a microparticle wherein the polymer is PLG of molecular weight less than 40kD.
21. A composition comprising a mixture of microparticles of less than 10 microns in diameter which comprise an external polymer shell and an internal aqueous solution of a bioactive agent, said composition comprising a first microparticle wherein the polymer is PLG of molecular weight less than 40kD and a second microparticle wherein the polymer is PLG of molecular weight more than 80kD.
22. A composition according to any of Claims 19 to 21 wherein the bioactive agent is selected from a protein antigen, a non-protein antigen, protein-or peptide-conjugated polysacharrides, protein-for peptide-DNA complexes, synthetic peptides, synthetic proteins, DNA viruses, cytokines, cancer therapeutics, mini-genes and water-soluble pharmaceuticals. The

- 37 -

bioactive agent may further be accompanied by excipient and/or adjuvant, one example of which is alum.

23. A composition according to any of Claims 19 to 21 wherein the bioactive agent is DNA comprising a coding sequence and wherein the microparticle when administered orally induces expression of the coding sequence.
24. A method of making a microparticle for use as or in a vaccinating composition, comprising following the method according to any of Claims 1 to 18, wherein the microparticle contains DNA coding for an antigen.
25. A composition for use as or in a vaccinating composition, comprising a composition according to any of Claims 19 to 23 wherein the microparticle contains DNA coding for an antigen.
26. Use of a composition according to any of Claims 19 to 23 in manufacture of a medicament for vaccination, wherein the microparticle contains DNA coding for an immunogen.
27. A method for encapsulating a bioactive agent in a polymer microparticle, comprising:
 - dissolving polymer in a solvent to form a polymer solution;
 - preparing an aqueous solution of a bioactive agent;
 - combining the polymer and aqueous solutions with agitation to form a water-in-oil emulsion;
 - adding the water-in-oil emulsion to a further aqueous phase with agitation to form a (water-in-oil)-in-water emulsion;
 - adding the (water-in-oil)-in-water emulsion to excess of an aqueous phase to extract the solvent, thereby forming microparticles of a size up to 10 microns in diameter, said microparticles containing bioactive agent in aqueous solution;

- 38 -

wherein the agitation steps comprise agitating the solutions or emulsions in the blending compartment of a blender, and using a blending blade which both blends and mixes the contents of the blending compartment.

28. A method according to Claim 27 wherein the blending is continued for a prolonged period of time so as to obtain microparticles the majority of which are of a size up to 10 microns in diameter.
29. A method for encapsulating a bioactive agent in a polymer microparticle, comprising:
 - dissolving polymer in a solvent to form a polymer solution;
 - preparing an aqueous solution of a bioactive agent;
 - combining the polymer and aqueous solutions with agitation to form a water-in-oil emulsion;
 - adding the water-in-oil emulsion to a further aqueous phase with agitation to form a (water-in-oil)-in-water emulsion;
 - adding the (water-in-oil)-in-water emulsion to excess of an aqueous phase to extract the solvent, thereby forming microparticles of a size up to 10 microns in diameter, said microparticles containing bioactive agent in aqueous solution;
 - wherein the polymer comprises or consists of PLG of molecular weight 40kD or lower.
30. A method for encapsulating a bioactive agent in a polymer microparticle, comprising:
 - dissolving polymer in a solvent to form a polymer solution;
 - preparing an aqueous solution of a bioactive agent;
 - combining the polymer and aqueous solutions with agitation to form a water-in-oil emulsion;
 - adding the water-in-oil emulsion to a further aqueous phase with agitation to form a (water-in-oil)-in-water emulsion;
 - adding the (water-in-oil)-in-water emulsion to excess of an aqueous phase

- 39 -

to extract the solvent, thereby forming microparticles of a size up to 10 microns in diameter, said microparticles containing bioactive agent in aqueous solution;

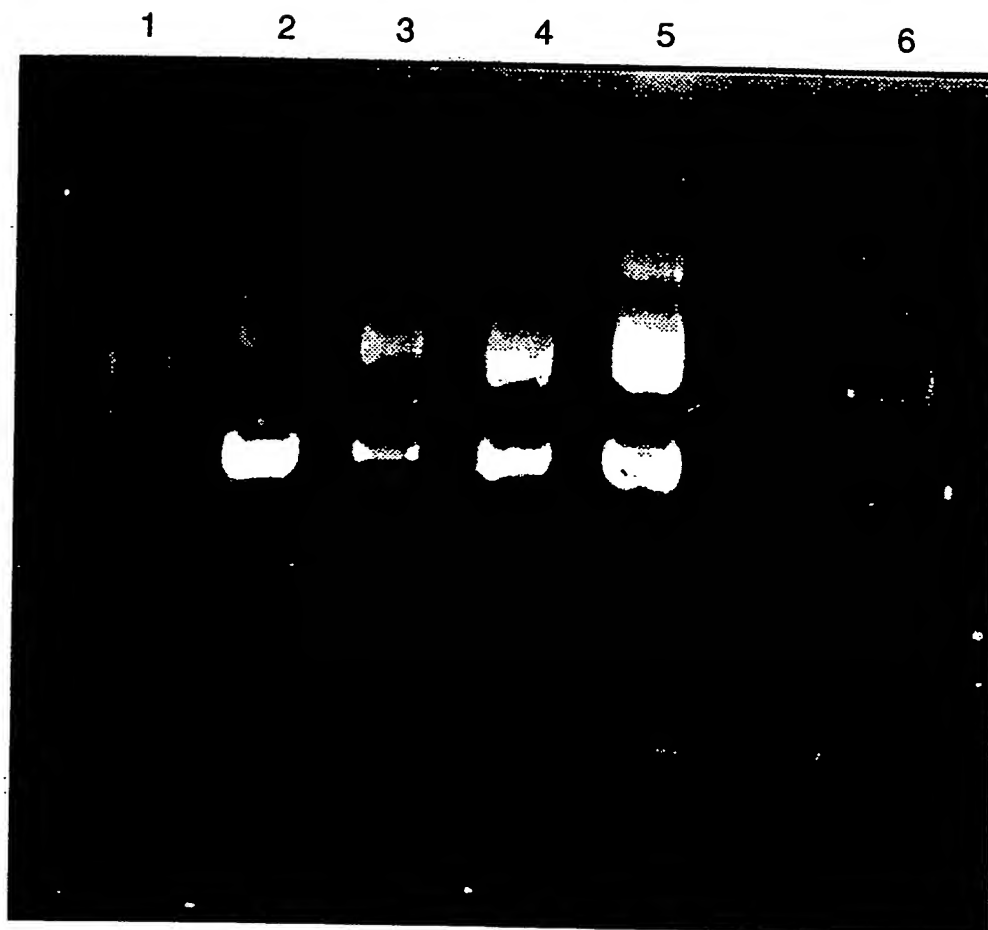
wherein the polymer comprises or consists of PLG of molecular weight 80kD or higher.

31. A microparticle obtainable according to the method of any of Claims 1 to 9.
32. A microparticle obtainable according to the method of any of Claims 10 to 18.
33. A microparticle obtainable according to the method of any of Claims 27 to 28.
34. A microparticle obtainable according to the method of Claim 29.
35. A microparticle obtainable according to the method of Claim 30.
36. A polymer microparticle, of size up to 10 microns in diameter, and containing a bioactive agent, wherein the polymer comprises PLG of inherent viscosity less than 0.5 dl/g.
37. A microparticle according to claim 36 wherein the bioactive agent is DNA comprising a coding sequence.
38. A microparticle according to claims 36 and 37 wherein the inherent viscosity of the PLG is in the range of 0.1 to 0.4 dl/g
39. A microparticle according to any of claims 36 to 38, wherein at least 30% of the bioactive agent is released within 20 days following administration.

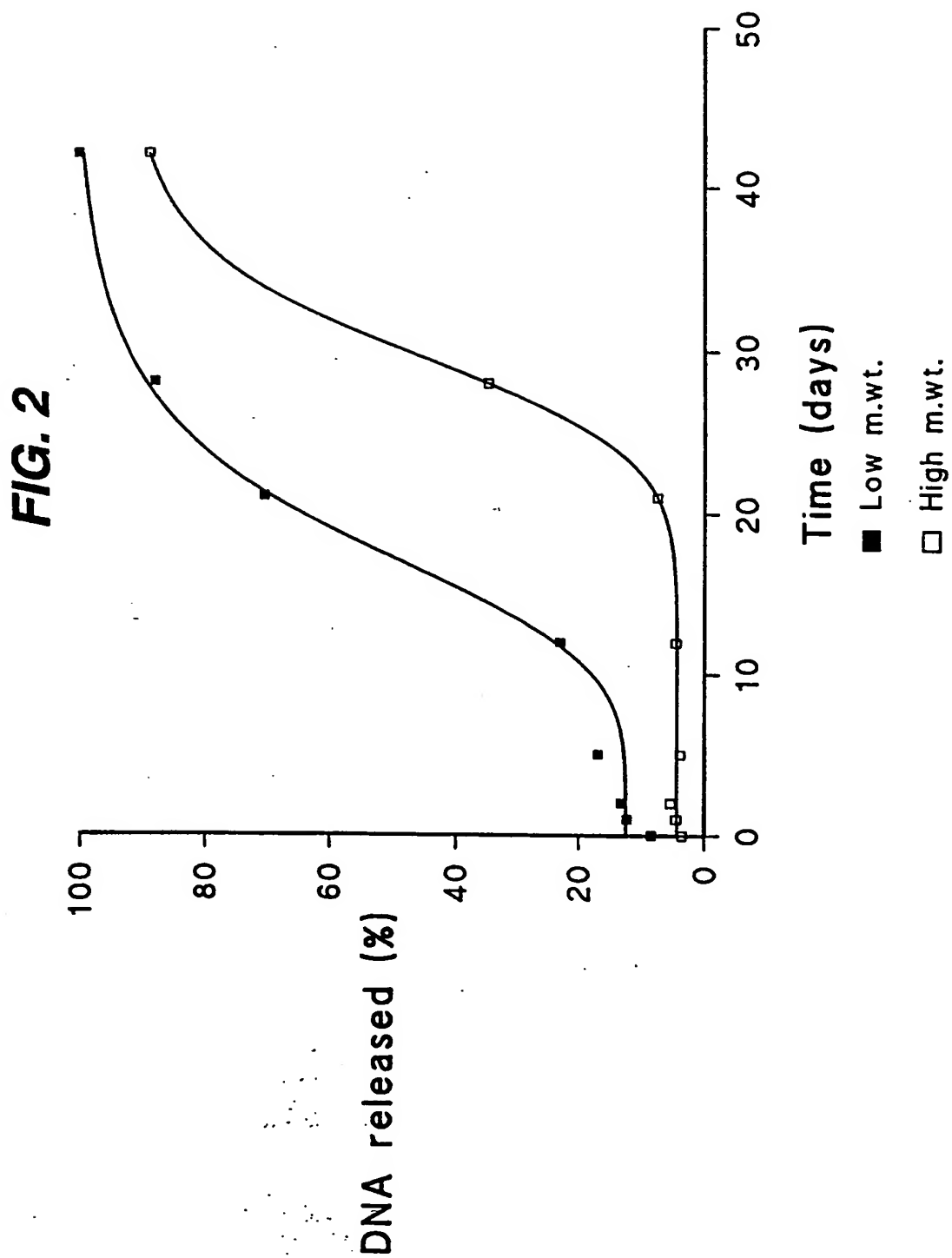
- 40 -

40. A microparticle according to any of claims 36 to 38, wherein at least 30% of the bioactive agent is released within 10 days following administration.
41. A method of encapsulating a bioactive agent in a polymer microparticle comprising combining in an apparatus an aqueous solution of said bioactive agent with a solution of a polymer in order to form a (water-in-oil)-in water emulsion, wherein said apparatus comprises:
- a container for a liquid to be mixed;
- a blade for mixing liquid in the container;
- wherein the blade rotates about an axis of rotation and exerts a mixing action that includes driving liquid in a direction substantially along the axis of rotation, and thereby facilitates the formation of a (water-in-oil)-in water emulsion.
42. A method according to claim 41 wherein the blade comprises a plurality of blade end tips that extend upwardly and downwardly from the blade.
43. A method according to claim 42 wherein the blade end tips are inclined between 30° and 60° relative to the blade surface.

FIG. 1



2/5



3/5

FIG. 3a

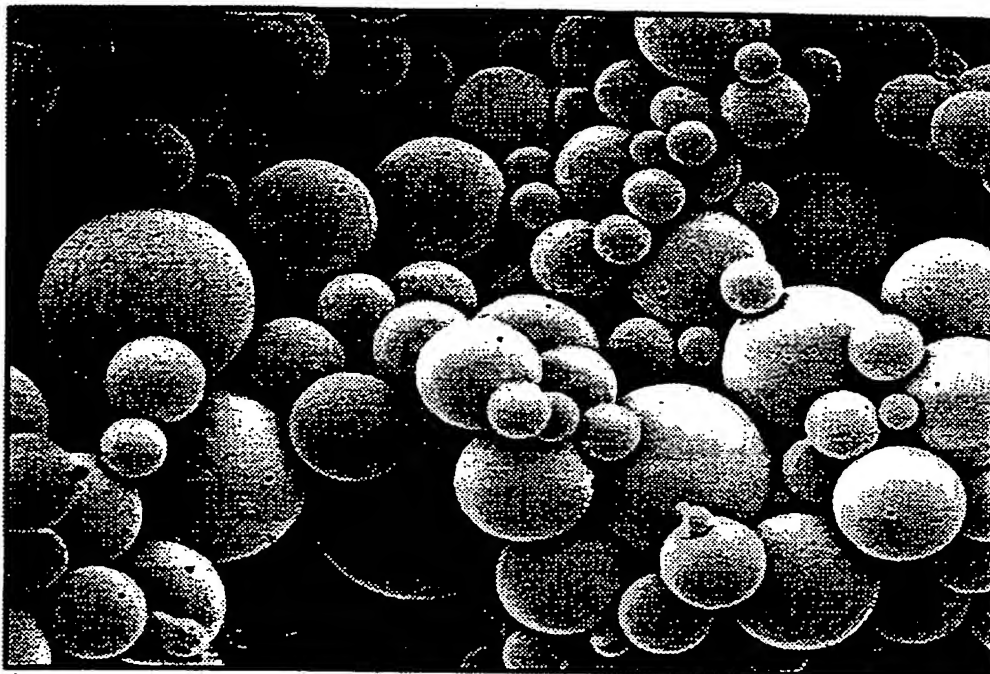
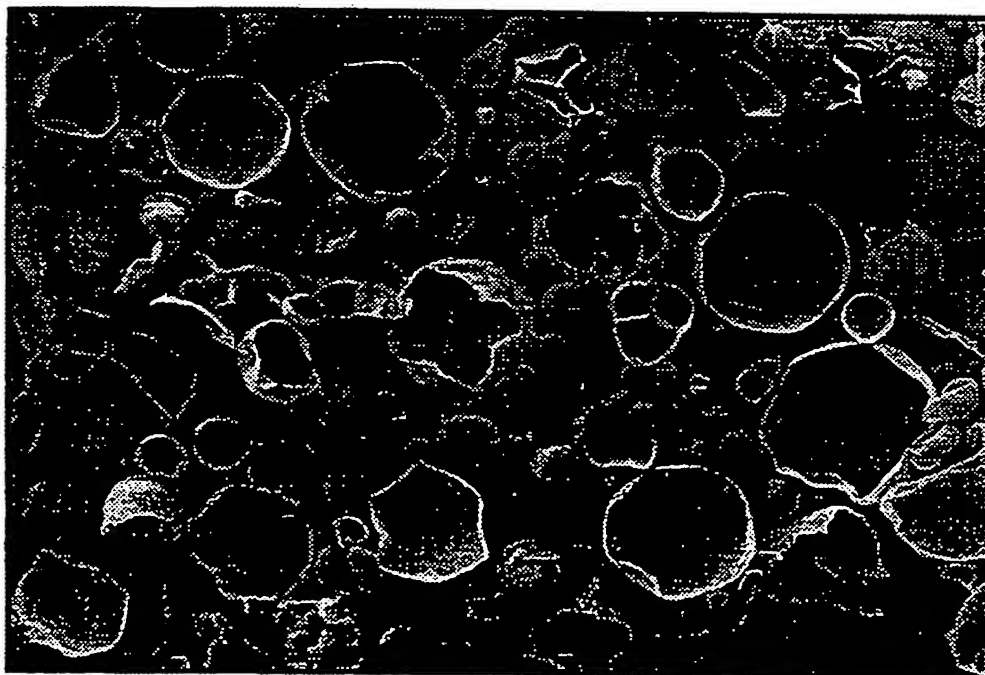


FIG. 3b



4/5

FIG. 4a

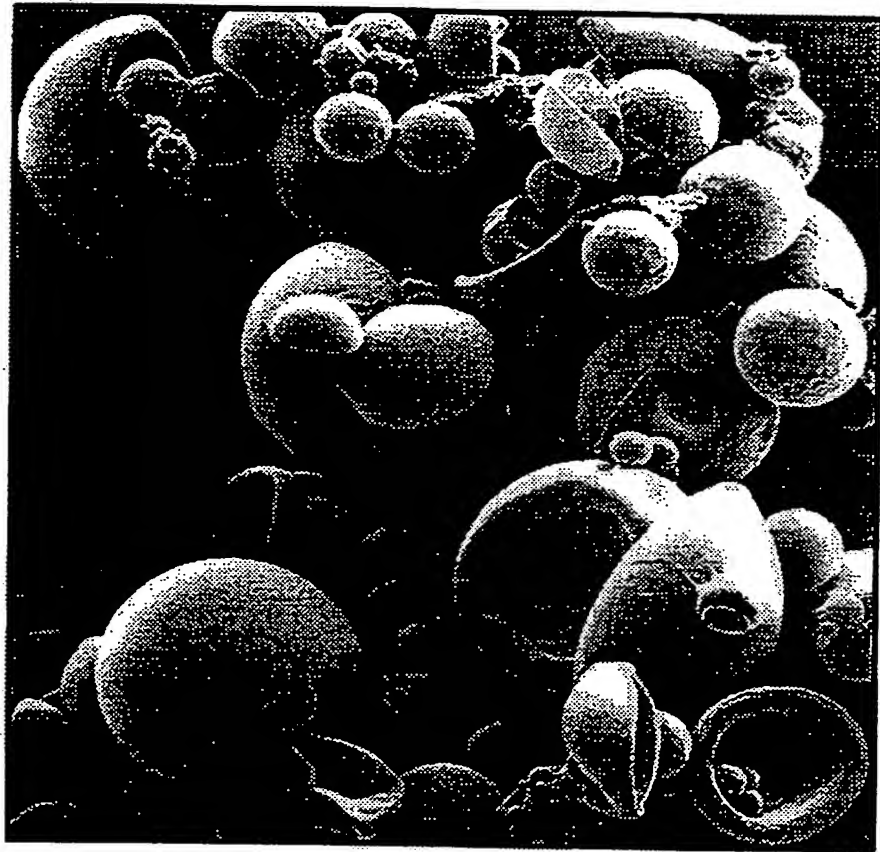
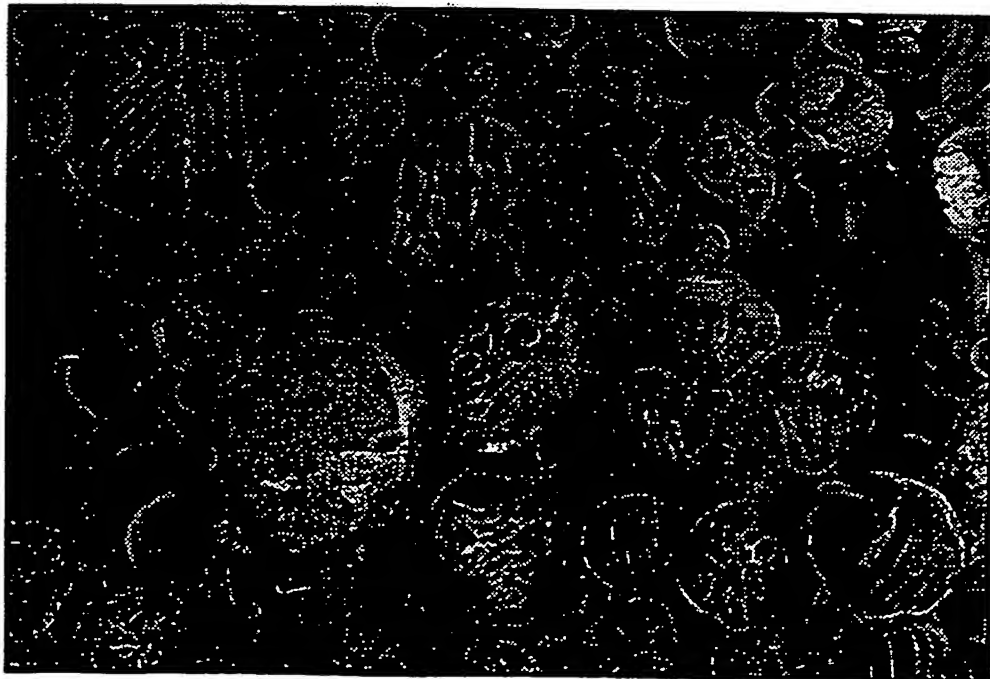
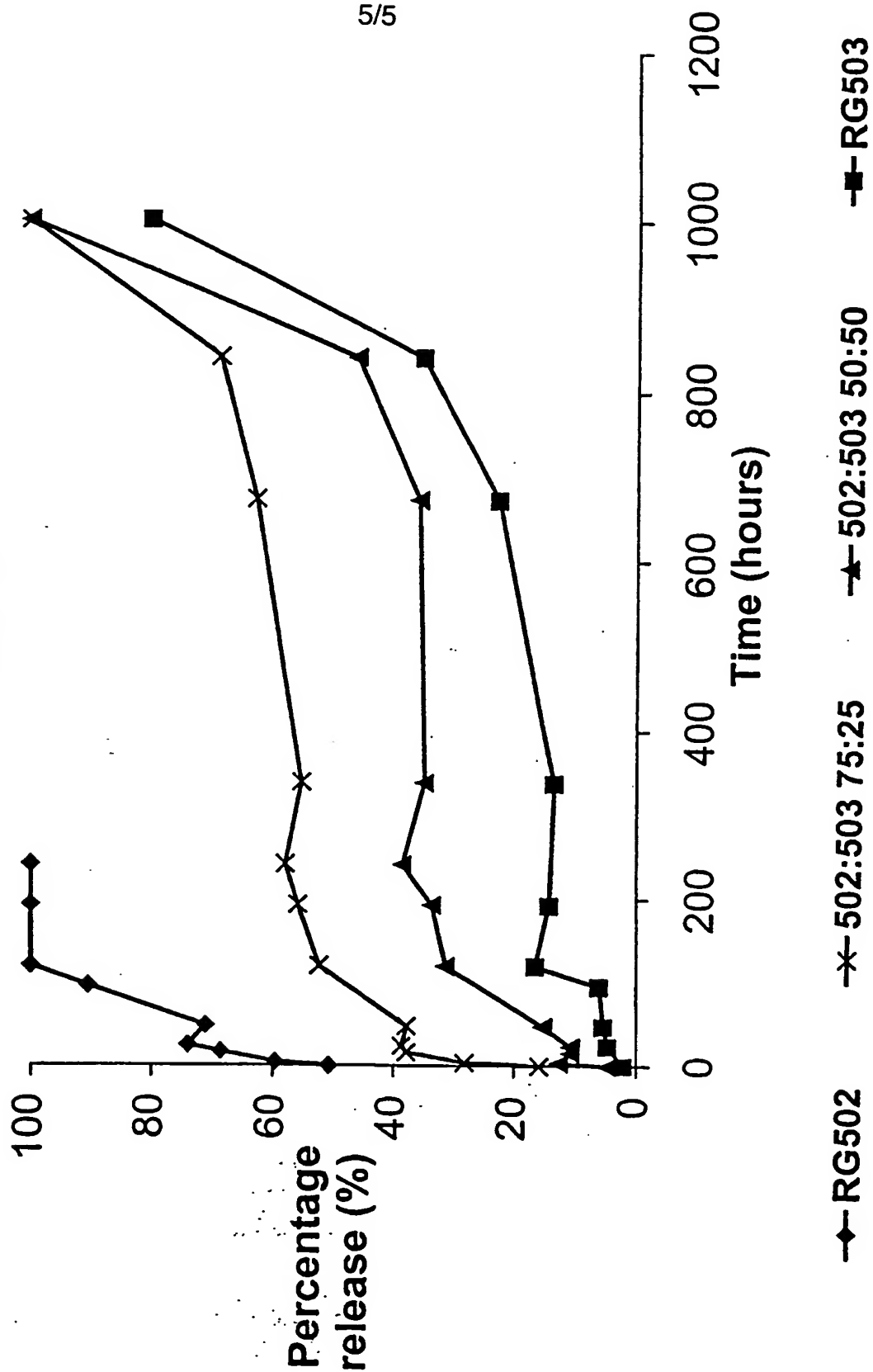


FIG. 4b(PRIOR ART)



5/5

FIG. 5



INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01449

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/16 A61K9/51

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X, Y L	WO 98 51279 A (FARRAR GRAHAM HENRY ; MICROBIOLOGICAL RES AUTHORITY (GB); CLEGG JAM) 19 November 1998 (1998-11-19) "L": DOCUMENT SO QUOTED FOR ITS' CASTING DOUBT ON THE VALIDITY OF THE CONVENTION-PRIORITY CLAIM. the whole document	1-43
X, Y	WO 95 11009 A (GENENTECH INC) 27 April 1995 (1995-04-27) the whole document	1-43
X, Y	EP 0 263 490 A (CHUGAI PHARMACEUTICAL CO LTD) 13 April 1988 (1988-04-13) the whole document	1-43
	--- -/-- ---	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"S" document member of the same patent family

Date of the actual completion of the international search

24 August 1999

Date of mailing of the international search report

01/09/1999

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3018

Authorized officer

Fischer, W

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01449

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y	GB 2 310 801 A (MERCK & CO INC) 10 September 1997 (1997-09-10) the whole document	1-43
Y	PATENT ABSTRACTS OF JAPAN vol. 018, no. 209 (C-1190), 13 April 1994 (1994-04-13) & JP 06 009377 A (UNITIKA LTD), 18 January 1994 (1994-01-18) abstract	1
A	JONES ET AL: "ORAL DELIVERY OF MICRO-ENCAPSULATED DNA VACCINES" DEVELOPMENTS IN BIOLOGICAL STANDARDIZATION, vol. 92, 18 June 1996 (1996-06-18), pages 149-155, XP002082346	
A	NELLORE R ET AL: "APPLICATION OF BIODEGRADABLE MICROSPHERES TO HEPATITIS B SURFACE ANTIGEN VACCINATION SYSTEM" PHARMACEUTICAL RESEARCH, vol. 8, 1 January 1991 (1991-01-01), page S151 XP002026578 ISSN: 0724-8741	
A	JONES ET AL: "POLY (DL-LACTIDE-CO- GLYCOLIDE)-ENCAPSULATED PLASMID DNA ELICITS SYSTEMIC AND MUCOSAL ANTIBODY RESPONSES TO ENCODED PROTEIN AFTER ORAL ADMINISTRATION" VACCINE, vol. 8, no. 15, 1 June 1997 (1997-06-01), page 814 817 XP002078102 ISSN: 0264-410X	
A	JONES ET AL: "ORAL DELIVERY OF POLY(LACTIDE-CO-GLYCOLIDE) ENCAPSULATED VACCINES" BEHRING INSTITUTE: MITTEILUNGEN, no. 98, 1 February 1997 (1997-02-01), page 220 228 XP002078100	
A	WO 97 17063 A (FARRAR GRAHAM HENRY ;MICROBIOLOGICAL RES AUTHORITY (GB); CLEGG JAM) 15 May 1997 (1997-05-15) cited in the application	
A	WO 97 35563 A (KUROKAWA TOMOFUMI ;IWASA SUSUMU (JP); TAKADA SHIGEYUKI (JP); TAKED) 2 October 1997 (1997-10-02)	
A	GB 2 234 896 A (SANDOZ LTD) 20 February 1991 (1991-02-20)	
	-/-	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 99/01449

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 2 265 311 A (SANDOZ LTD) 29 September 1993 (1993-09-29)	
A	US 4 652 441 A (YASHIKI TAKATSUKA ET AL) 24 March 1987 (1987-03-24)	
A	US 4 711 782 A (YASHIKI TAKATSUKA ET AL) 8 December 1987 (1987-12-08)	
A	US 5 650 173 A (RAMSTACK J MICHAEL ET AL) 22 July 1997 (1997-07-22)	
A	PATENT ABSTRACTS OF JAPAN vol. 017, no. 158 (C-1041), 29 March 1993 (1993-03-29) & JP 04 321622 A (TAKEDA CHEM IND LTD), 11 November 1992 (1992-11-11) abstract	
A	EP 0 779 072 A (TAKEDA CHEMICAL INDUSTRIES LTD) 18 June 1997 (1997-06-18)	
A	EP 0 145 240 A (TAKEDA CHEMICAL INDUSTRIES LTD) 19 June 1985 (1985-06-19)	
A	US 4 853 226 A (MACHIDA MINORU ET AL) 1 August 1989 (1989-08-01)	
A	US 4 917 893 A (YASHIKI TAKATSUKA ET AL) 17 April 1990 (1990-04-17)	
A	US 4 933 105 A (FONG JONES W) 12 June 1990 (1990-06-12)	
A	US 5 061 492 A (YASHIKI TAKATSUKA ET AL) 29 October 1991 (1991-10-29)	
A	US 5 540 937 A (TEICHNER MARC M ET AL) 30 July 1996 (1996-07-30)	
A	US 5 654 008 A (HERBERT PAUL F ET AL) 5 August 1997 (1997-08-05)	

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9851279 A	19-11-1998	AU 7440898 A	08-12-1998
WO 9511009 A	27-04-1995	AT 175110 T	15-01-1999
		AU 8017494 A	08-05-1995
		CA 2172508 A	27-04-1995
		DE 69415684 D	11-02-1999
		DE 69415684 T	10-06-1999
		EP 0724433 A	07-08-1996
		JP 9504026 T	22-04-1997
EP 0263490 A	13-04-1988	JP 7025689 B	22-03-1995
		JP 63091325 A	22-04-1988
		AT 116540 T	15-01-1995
		AU 608250 B	28-03-1991
		CA 1315200 A	30-03-1993
		DE 3750943 D	16-02-1995
		DE 3750943 T	11-05-1995
		ES 2068810 T	01-05-1995
		GR 3015485 T	30-06-1995
		US 4853226 A	01-08-1989
GB 2310801 A	10-09-1997	NONE	
JP 06009377 A	18-01-1994	NONE	
WO 9717063 A	15-05-1997	AU 7578996 A	29-05-1997
		CA 2236925 A	15-05-1997
		EP 0862419 A	09-09-1998
WO 9735563 A	02-10-1997	AU 2043297 A	17-10-1997
		CA 2241322 A	02-10-1997
		EP 0889722 A	13-01-1999
		JP 9315997 A	09-12-1997
GB 2234896 A	20-02-1991	AT 144090 A	15-08-1999
		AU 687553 B	26-02-1998
		AU 2332195 A	07-09-1995
		AU 4198593 A	23-09-1993
		AU 4198693 A	23-09-1993
		AU 641407 B	23-09-1993
		AU 5874690 A	10-01-1991
		BE 1004486 A	01-12-1992
		CA 2020477 A	08-01-1991
		CH 685230 A	15-05-1995
		CH 686226 A	15-02-1996
		CH 686252 A	15-02-1996
		CY 1844 A	08-03-1996
		CY 1965 A	04-07-1997
		DE 4021517 A	17-01-1991
		DK 162590 A	08-01-1991
		FI 991120 A	17-05-1999
		FR 2649319 A	11-01-1991
		GB 2265311 A, B	29-09-1993
		GR 90100513 A, B	10-12-1991
		HK 97695 A	23-06-1995
		HK 197496 A	08-11-1996
		HU 9500523 A	30-10-1995
		IE 64216 B	26-07-1995

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2234896 A		IE 64411 B	09-08-1995
		IT 1241460 B	17-01-1994
		JP 7285853 A	31-10-1995
		JP 7309897 A	28-11-1995
		JP 2112513 C	21-11-1996
		JP 3068511 A	25-03-1991
		JP 8032624 B	29-03-1996
		JP 8198771 A	06-08-1996
		LU 87764 A	11-03-1992
		NL 9001537 A	01-02-1991
		NO 302928 B	11-05-1998
		NO 960075 A	08-01-1991
		NO 960076 A	08-01-1991
		NO 983923 A	08-01-1991
		NZ 234384 A	26-05-1994
		PT 94628 A,B	20-03-1991
		SE 9002364 A	08-01-1991
		SG 9590737 A	01-09-1995
		US 5639480 A	17-06-1997
		US 5876761 A	02-03-1999
		US 5688530 A	18-11-1997
		US 5538739 A	23-07-1996
GB 2265311 A	29-09-1993	AT 144090 A	15-08-1999
		AU 687553 B	26-02-1998
		AU 2332195 A	07-09-1995
		AU 4198593 A	23-09-1993
		AU 4198693 A	23-09-1993
		AU 641407 B	23-09-1993
		AU 5874690 A	10-01-1991
		BE 1004486 A	01-12-1992
		CA 2020477 A	08-01-1991
		CH 685230 A	15-05-1995
		CH 686226 A	15-02-1996
		CH 686252 A	15-02-1996
		CY 1844 A	08-03-1996
		CY 1965 A	04-07-1997
		DE 4021517 A	17-01-1991
		DK 162590 A	08-01-1991
		FI 991120 A	17-05-1999
		FR 2649319 A	11-01-1991
		GB 2234896 A,B	20-02-1991
		GR 90100513 A,B	10-12-1991
		HK 97695 A	23-06-1995
		HK 197496 A	08-11-1996
		HU 9500523 A	30-10-1995
		IE 64216 B	26-07-1995
		IE 64411 B	09-08-1995
		IT 1241460 B	17-01-1994
		JP 7285853 A	31-10-1995
		JP 7309897 A	28-11-1995
		JP 2112513 C	21-11-1996
		JP 3068511 A	25-03-1991
		JP 8032624 B	29-03-1996
		JP 8198771 A	06-08-1996
		LU 87764 A	11-03-1992
		NL 9001537 A	01-02-1991
		NO 302928 B	11-05-1998

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 99/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2265311 A		NO 960075 A	08-01-1991
		NO 960076 A	08-01-1991
		NO 983923 A	08-01-1991
		NZ 234384 A	26-05-1994
		PT 94628 A,B	20-03-1991
		SE 9002364 A	08-01-1991
		SG 9590737 A	01-09-1995
		US 5639480 A	17-06-1997
		US 5876761 A	02-03-1999
		US 5688530 A	18-11-1997
		US 5538739 A	23-07-1996
US 4652441 A	24-03-1987	JP 1057087 B	04-12-1989
		JP 1761586 C	28-05-1993
		JP 60100516 A	04-06-1985
		AT 42197 T	15-05-1989
		BG 60493 B	31-05-1995
		CA 1233414 A	01-03-1988
		EP 0145240 A	19-06-1985
		GR 80818 A	04-03-1985
		HK 3792 A	17-01-1992
		IE 57721 B	24-03-1993
		LV 5755 A	20-12-1996
		PT 79450 A,B	01-12-1984
		US 4917893 A	17-04-1990
		US 5476663 A	19-12-1995
		US 5631020 A	20-05-1997
		US 5061492 A	29-10-1991
		US 5631021 A	20-05-1997
		US 4711782 A	08-12-1987
US 4711782 A	08-12-1987	JP 1057087 B	04-12-1989
		JP 1761586 C	28-05-1993
		JP 60100516 A	04-06-1985
		AT 42197 T	15-05-1989
		BG 60493 B	31-05-1995
		CA 1233414 A	01-03-1988
		EP 0145240 A	19-06-1985
		GR 80818 A	04-03-1985
		HK 3792 A	17-01-1992
		IE 57721 B	24-03-1993
		LV 5755 A	20-12-1996
		PT 79450 A,B	01-12-1984
		US 4917893 A	17-04-1990
		US 5476663 A	19-12-1995
		US 5631020 A	20-05-1997
		US 5061492 A	29-10-1991
		US 5631021 A	20-05-1997
		US 4652441 A	24-03-1987
US 5650173 A	22-07-1997	AU 684324 B	11-12-1997
		AU 1101095 A	06-06-1995
		AU 697887 B	22-10-1998
		AU 3683197 A	20-11-1997
		CA 2176716 A	26-05-1995
		EP 0729353 A	04-09-1996
		JP 9505308 T	27-05-1997
		WO 9513799 A	26-05-1995

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/G8 99/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4853226 A		ES 2068810 T GR 3015485 T	01-05-1995 30-06-1995
US 4917893 A	17-04-1990	JP 1057087 B JP 1761586 C JP 60100516 A AT 42197 T BG 60493 B CA 1233414 A EP 0145240 A GR 80818 A HK 3792 A IE 57721 B LV 5755 A PT 79450 A, B US 5476663 A US 5631020 A US 5061492 A US 5631021 A US 4652441 A US 4711782 A	04-12-1989 28-05-1993 04-06-1985 15-05-1989 31-05-1995 01-03-1988 19-06-1985 04-03-1985 17-01-1992 24-03-1993 20-12-1996 01-12-1984 19-12-1995 20-05-1997 29-10-1991 20-05-1997 24-03-1987 08-12-1987
US 4933105 A	12-06-1990	US 4384975 A CH 648217 A DE 3121983 A FR 2484281 A GB 2077693 A, B IT 1171290 B JP 57027128 A	24-05-1983 15-03-1985 04-02-1982 18-12-1981 23-12-1981 10-06-1987 13-02-1982
US 5061492 A	29-10-1991	JP 1057087 B JP 1761586 C JP 60100516 A AT 42197 T BG 60493 B CA 1233414 A EP 0145240 A GR 80818 A HK 3792 A IE 57721 B LV 5755 A PT 79450 A, B US 4917893 A US 5476663 A US 5631020 A US 5631021 A US 4652441 A US 4711782 A	04-12-1989 28-05-1993 04-06-1985 15-05-1989 31-05-1995 01-03-1988 19-06-1985 04-03-1985 17-01-1992 24-03-1993 20-12-1996 01-12-1984 17-04-1990 19-12-1995 20-05-1997 20-05-1997 24-03-1987 08-12-1987
US 5540937 A	30-07-1996	FR 2693905 A AU 675788 B AU 4202293 A CA 2100925 A EP 0585151 A JP 6087758 A NZ 248207 A	28-01-1994 20-02-1997 10-02-1994 28-01-1994 02-03-1994 29-03-1994 27-02-1996
US 5654008 A	05-08-1997	AU 684324 B	11-12-1997

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/GB 99/01449

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5654008 A		AU 1101095 A	06-06-1995
		AU 697887 B	22-10-1998
		AU 3683197 A	20-11-1997
		CA 2176716 A	26-05-1995
		EP 0729353 A	04-09-1996
		JP 9505308 T	27-05-1997
		WO 9513799 A	26-05-1995
		US 5650173 A	22-07-1997